Pergamon

Tetrahedron 54 (1998) 1657–1666

TETRAHEDRON

New Ergostane Type Ecdysteroids from Fungi. Ecdysteroid Constituents of Mushroom *Paxillus atrotomentosus* [1]

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Received 14 October 1997; revised 17 November 1997; accepted 20 November 1997

Abstract: New ergostane type ecdysteroids from the mushroom species *Paxillus atrotomentosus*: paxillosterone, its 20,22-*p*-hydroxybenzylidene acetal, atrotosterones A, B and C and 25-hydroxy-atrotosterones A and B have been characterized. 20-Hydroxyecdysone as a minor constituent has also been isolated. Configuration at C(24) of paxillosterone was derived from proton 2D-ROESY NMR spectra of its cyclic phenylboronate derivatives. Configuration at C(24) of 25-hydroxyatrotosterone A was assigned by comparison of its NMR spectra with the spectra of both 24-epimers of makisterone A.

INTRODUCTION

Since the first isolation and identification of phytoecdysteroids, compounds structurally related to the insect moulting hormone ecdysone from plants, a considerable effort to ascertain their possible significance and role in the plant - insect chemical interaction has been expended [2]. In spite of this effort there is no direct evidence that these compounds can take part in any kind of known protection mechanism [3]. However, the idea that ecdysteroids may play a role in plant defense against phytophagous insects seems to be generally accepted.

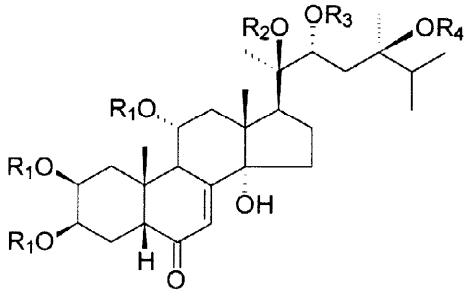
In the continuation of our previous research on phytoecdysteroids [1] we decided to look for the presence of ecdysteroids also in fungi (Mycophyta), particularly in those species which are generally not attacked by insects. Occurrence of some ecdysteroid-related compounds in fungi has been already reported [4,5], but their relation to insect moulting hormone activity has not been described. This fact probably retarded the interest in investigation of ecdysteroids in fungi up to the recent time. The discovery of paxillosterone (**1**), the major ecdysteroid constituent of the mushroom *Paxillus atrotomentosus* (Batsch) Fr. in our laboratory [6] initiated our interest in detailed analysis and in testing their biological activity [7]. In this paper we present our results on identification of eight ecdysteroids isolated from this mushroom.

RESULTS AND DISCUSSION

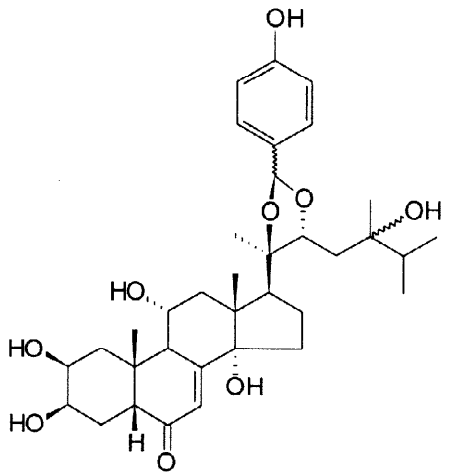
Ecdysteroids **1-8** were isolated from methanolic extract of *Paxillus atrotomentosus* by a separation procedure described in the experimental part. Structures of the major ecdysteroid constituent paxillosterone (**1**) and of minor constituents **2-7** were elucidated by NMR (for ^{1}H and ^{13}C NMR data see Tables 1 and 2), IR and mass spectrometry. Their structures are related to makisterone A, a 24-methyl homologue of ecdysone, *i.e.* they are of ergostane type. Because the configuration on C(24) could not be assigned in all new constituents, their names could not be derived from makisterone A. They had to receive new names.

0040-4020/98/$19.00
PII: S0040-4020(97)10373-8

The compound **8** was identified as 20-hydroxyecdysone, found in this species exceptionally as a minor constituent.



1 $R_1 = R_2 = R_3 = R_4 = H$
1a $R_1 = R_4 = H$, $R_2,R_3 = >C(CH_3)_2$
1b $R_1 = R_4 = H$, $R_2,R_3 = >BC_6H_5$
1c $R_1 = R_2 = H$, $R_3,R_4 = >BC_6H_5$
1d $R_1 = Ac$, $R_2,R_3 = >BC_6H_5$, $R_4 = H$
1e $R_1 = Ac$, $R_3,R_4 = >BC_6H_5$, $R_2 = H$



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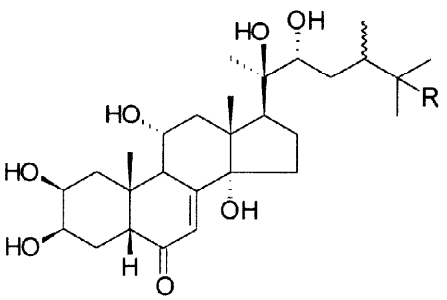
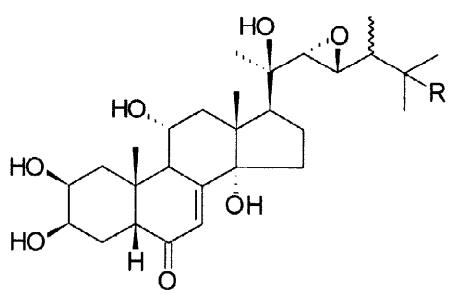
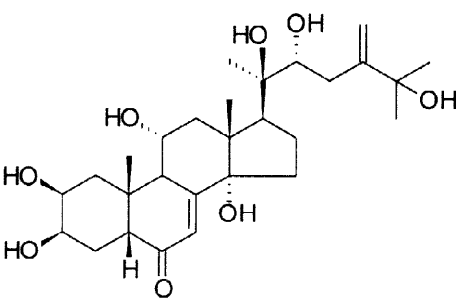


3 R = H
4 R = OH [24S]



5 R = H
6 R = OH



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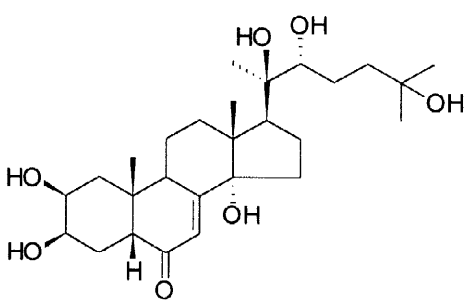

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For determination of the absolute configuration at C(24) of paxillosterone (**1**), preparation of a cyclic derivative in which carbon C(24) became a part of a conformationally fixed system was required. In such a system the spatial proximity of protons can be determined by NMR methods (NOE-effect). Various agents were utilised to produce a suitable cyclic derivative. Treatment of paxillosterone (**1**) with benzaldehyde using method described in the literature [8,9] led to a complex mixture of products. Paxillosterone acetonides prepared by a

common method [10] gave upon equilibration in protic solvent only the 20,22-acetonide **1a.** Finally, cyclic phenylboronate group was proved to give the most suitable results. Phenylboronic acid is generally used for protection of 1,2 and 1,3 diols. It forms rather stable cyclic esters with the side chain diols of ecdysteroids [11]. Phenylboronates of 20,22-dihydroxyecdysteroids were extensively used in our laboratory as selective protected intermediates for regiospecific synthesis of conjugates [12], as derivatives with specifically modified properties for HPLC [13] and FAB-MS analyses [14], or for solid phase extractions and chromatography separations [15].

Treatment of paxillosterone (**1**) with phenylboronic acid led to a mixture of 20,22- and 22,24-phenylboronates **1b** and **1c**. These two isomers were not separable on HPLC even when various sorbents and mobile phases were used. Moreover, their ratio was different in various solvents, *e.g.* 1:1 in methanol and 1:4 in chloroform (determined by NMR). Apparently, in the solution there is a dynamic equilibrium between five-membered and six-membered boronate rings. Although, the higher abundance of 22,24-phenylboronate **1c** in chloroform was favourable for determination of C(24) configuration, the spectra obtained in this solvent were not sufficiently resolved for detailed analysis of both components. However, acetylation of the boronate mixture gave 2,3,11-triacetates **1d** and **1e** (also as a mixture of two inseparable isomers) whose NMR spectra in $CDCl_3$ were well resolved and could be used for the assignment of configuration at C(24). The ratio of acetylated 20,22- and 22,24-phenyl- boronate isomers was the same as that of non-acetylated compounds in both methanol and chloroform.

The 1D and 2D-COSY spectra were used for structural assignment of protons. Vicinal coupling constants between H(22) and H(23a), H(23b) (12.0 and 3.0 Hz) indicated the axial position of H(22) in a six-membered ring of 22,24-phenylboronate **1e**. The observed cross-peaks of H(22) with H(25) and Me(26) in 2D-ROESY spectrum unequivocally proved the [24R] configuration in primary paxillosterone (**1**) (see Figure 1A) on condition that the C(22) configuration is [22R] as it is usual in all naturally occuring ecdysteroids. The configuration at position C(20) was confirmed by NOE cross-peaks in acetylated 20,22-phenylboronate **1d** and - more easily - from 2D-ROESY spectrum of 20,22-acetonide **1a**. The NOE contacts of proton Me(21) to both H(23a), H(23b) and the absence of cross-peak between Me(21) and H(22) (see Figure 1B) indicate *cis*-orientation of Me(21) and C(23) in agreement with configurations at C(20), C(22) in structure **1**.

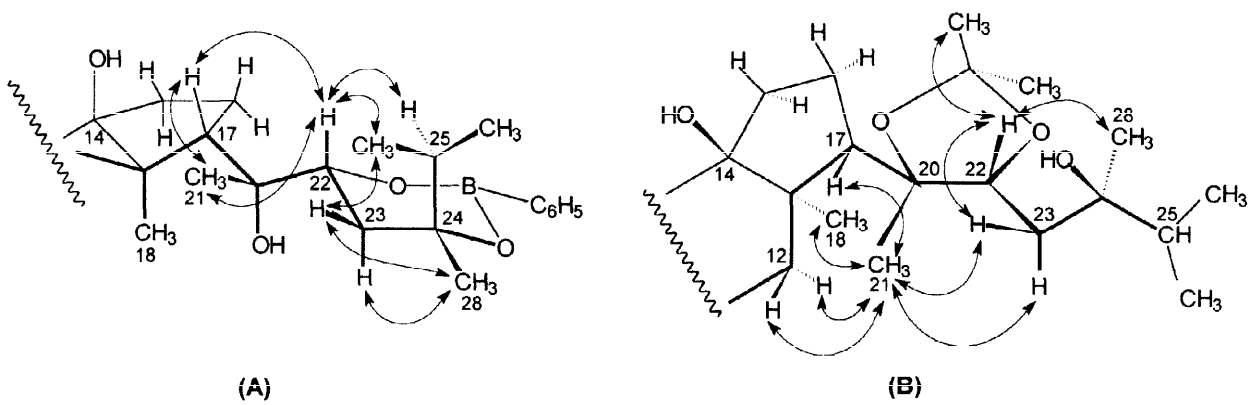

Figure 1 The observed NOE-contacts of side-chain protons in 2D-ROESY spectra of acetylated 22,24-phenyboronate **1e** (A) and 20,22-acetonide **1a** (B)

The presence of unusual *p*-hydroxybenzylidene acetal group in position 20,22 of compound **2** was manifested in ^{1}H NMR spectrum by the singlet of acetal proton at δ 5.73 and AA'XX' spin system of aromatic protons at δ 6.77 (2H) and 7.29 (2H). The substitution is accompanied with low-field shifts of neighbouring protons Me(21), H(22) and Me(28), (see Table 1).

Configuration at C(24) of 25-hydroxyatrotosterone A (**4**) was determined by comparison with NMR data of makisterone A and 24-*epi*-makisterone A (ref. [16]). Side-chain proton chemical shifts (see Table 1) are in a very good agreement with corresponding ones of 24-*epi*-makisterone A and indicate [24S]-configuration in 25-hydroxyatrotosterone A (**4**). The absence of 25-OH group in atrotosterone A (**3**) influences the chemical shifts of side-chain protons and therefore does not allow their use to determine the configuration at C(24). However, from the biogenetic relation of ecdysteroids the identical configuration at C(24) can be assumed.

The presence of epoxy group in atrotosterone B (**5**) and 25-hydroxyatrotosterone B (**6**) is evidenced from ^{1}H NMR and ^{13}C NMR spectra, *e.g.* in atrotosterone B (**5**) - H(22): δ 2.85 d, J = 2.4 Hz and H(23): δ 2.72 dd, J = 7.8 and 2.4 Hz; C(22): δ 66.75, C(23): δ 59.93. The value of J(22,23) = 2.4 Hz indicates *trans*-orientation of corresponding protons. Configuration at C(24) was not determined.

Atrotosterone C (**7**) has an exomethylene instead of the methyl group in position 24 (^{1}H NMR - C=CH$_2$ protons at δ 5.14 bs and δ 4.96 bs; ^{13}C NMR - olefinic carbons C(24): δ 155.34 and C(28): δ 110.42).

The presence of 11α-hydroxy group in compounds **1-7** is manifested in ^{1}H NMR spectra by characteristic multiplet of additional CH-O proton (H(11β) at δ ~ 4.10 ddd, J ~ 10.5; 9.0 and 6.0 Hz) and lowfield shift of H(1β) (δ ~2.59 in **1** - **7** in comparison with δ ~1.79 in **8**) apparently due to the electrostatic interaction with the sterically close 11α-OH group. ^{13}C NMR spectra of **1-7** contain additional CH-O carbon (C(11) at δ ~ 69.50) and characteristic lowfield shifts of neighbouring carbons C(9) and C(12), (see Table 2). The presence of 11α-OH group is characteristic for constituents of *P. atrotomentosus*, except of the minor 20-hydroxyecdysone (**8**). However, any generalization of its chemotaxonomic value for fungi can not be significant, because of its absence in the structurally related polyporusterones from *Polyporus umbellatus* [5]. More significant for mushrooms seems to be the 24-methyl homology (the presence of ergostane skeleton) in both cases, as well as in ecdysone related polyhydroxylated steroids of *Lasiosphaera nipponica* (Gasteromycetes) [4].

20-hydroxyecdysone (**8**), present in this species exceptionally as minor constituent, was identified by comparison with published NMR data [17] and by HPLC analysis using authentic sample.

EXPERIMENTAL

Infrared spectra were recorded on a Bruker IPS-88 using KBr pellet. NMR spectra were measued on a Varian UNITY-500 instrument (^{1}H at 500 MHz; ^{13}C at 125.7 MHz) in CD$_3$OD (compounds **1** - **8**) or CDCl$_3$ (mixture of **1d** and **1e**). Chemical shifts in CD$_3$OD were referenced to the solvent signal at 3.31 ppm (^{1}H) and 49.00 ppm (^{13}C); in CDCl$_3$ to internal tetramethylsilane at 0 ppm (^{1}H) and solvent signal at 77.0 ppm (^{13}C). Proton 2D-COSY spectra [18] (with second pulse 45°) were measured for structural assignment of protons. Proton 2D-ROESY spectra [19] of acetonide **1a** and the mixture of acetylated phenyboronates **1d, 1e** were acquired with a spin-lock time 0.25 s. Carbon-13 spectra were run using the APT pulse sequence [20]. Mass spectra were recorded on a ZAB-EQ spectrometer with fast atom bombardment (FAB) ionisation using a glycerol - thioglycerol mixture as a matrix. Electron-impact mass spectra (EI-MS) were also recorded for most of new compounds. The melting points were determined on a Boëtius apparatus and are uncorrected.

Table 1. Proton NMR Data of Ecdysteroids **1 - 8** from *Paxillus atrotomentosus* in CD_3OD

Proton	Chemical shifts (ppm) / *Coupling constants (Hz)*							
	1	**2** [a]	**3**	**4**	**5**	**6**	**7** [b]	**8** [c]
H-1α	2.58 dd *12.5; 4.0*	2.59 dd *12.8; 4.2*	2.59 dd *12.8; 4.2*	2.59 dd *13.0; 4.2*	2.59 dd *12.8; 4.2*	2.59 dd *12.8; 4.2*	2.59 dd *12.8; 4.2*	1.79 dd *13.3; 4.6*
H-1β	1.37 dd *12.5; 11.8*	1.38 dd *13.0; 12.3*	1.38 dd *12.8; 12.0*	1.38 dd *13.0; 12.0*	1.38 dd *12.8; 12.0*	1.37 t *12.5; 12.5*	1.38 dd *12.8; 12.0*	1.43 dd *13.3; 12.0*
H-2α	4.00 ddd *11.8; 4.0; 2.6*	4.01 ddd *11.7; 4.0; 3.3*	4.01 ddd *11.0; 4.2; 3.3*	4.01 ddd *12.0; 4.2; 3.5*	4.01 ddd *11.7; 4.0; 3.3*	4.01 dt *11.8; 3.6; 3.6*	4.01 ddd *11.3; 4.8; 3.0*	3.84 ddd *12.0; 4.0; 3.2*
H-3α	3.95 q *2.6*	3.96 q *2.8*	3.95 q *3.0*	3.96 q *2.8*	3.96 q *2.9*	3.96 q *2.8*	3.95 q *~3.0*	3.95 q *~2.9*
H-4α	1.78 ddd *14.0; 12.8; 2.6*	1.79 dt *14.2; 13.2; 2.5*	1.77 ddd *14.0; 13.0; 2.5*	1.78 td *13.5; 13.5; 2.5*	1.78 dt *13.5; 13.5; 2.4*	1.78 dt *13.6; 13.6; 2.4*	1.78 dt *13.0; 13.0; 2.5*	1.78 *
H-4β	1.68 ddd	1.69 dt *14.2; 4.0; 3.5*	1.69 dt *14.0; 3.6; 3.6*	1.68 dt *14.3; 3.2; 3.2*	1.69 dt *14.0; 3.6; 3.6*	1.69 dt *14.0; 3.7; 3.7*	1.69 dt *14.0; 3.7; 3.7*	1.69 *
H-5	2.33 dd *12.8; 4.0*	2.34 dd *13.2; 3.8*	2.33 dd *13.0; 4.0*	2.34 dd *13.2; 4.0*	2.34 dd *13.0; 4.0*	2.34 dd *13.2; 4.0*	2.34 dd *13.0; 3.8*	2.38 dd *13.0; 4.5*
H-7	5.80 dd *2.7; 0.7*	5.82 dd *2.7; 0.8*	5.80 dd *2.7; 1.0*	5.80 dd *2.7; 0.8*	5.81 dd *2.7; 1.0*	5.81 d *2.5*	5.81 bd *2.7*	5.81 d *2.5*
H-9	3.13 dd *8.9; 2.7*	3.15 dd *9.0; 2.7*	3.16 dd *9.0; 2.7*	3.14 dd *8.8; 2.7*	3.16 dd *9.0; 2.7*	3.16 dd *9.0; 2.6*	3.15 dd *8.8; 2.8*	3.15 ddd *11.2; 7.0; 2.5*
H-11β	4.09 bddd *10.3; 8.9; 6.3*	4.10 ddd *10.7; 8.7; 6.0*	4.10 ddd *10.6; 9.0; 6.0*	4.10 ddd *10.5; 8.6; 6.0*	4.10 ddd *10.7; 8.7; 6.0*	4.10 ddd *10.5; 9.0; 6.0*	4.11 ddd *10.5; 9.0; 6.0*	1.69 *
H-12α	2.19 dd *12.5; 10.3*	2.22 dd *12.2; 10.8*	2.21 dd *12.1; 10.7*	2.21 dd *11.8; 10.7*	2.25 dd *12.0; 11.0*	2.25 t *~12.0; ~11.0*	2.23 dd *12.0; 10.5*	2.13 dt *13.0; 13.0; 4.8*
H-12β	2.14 dd *12.5; 6.3*	2.14 dd *12.0; 6.0*	2.15 dd *12.1; 6.0*	2.15 dd *12.2; 6.0*	2.14 dd *12.2; 5.8*	2.14 dd *12.2; 5.8*	2.17 dd *12.0; 6.2*	1.88 ddd *13.0; 4.6; 2.3*
H-15α	1.95 *	~2.05 *	1.95 *	1.95 *	~1.96 *	1.96 *	1.97 m	1.99 *
H-15β	1.56 *	1.63 m	1.58 *	1.57 *	1.62 m	1.61 *	1.59 m	1.60 m
H-16α	2.01 *	~2.05 *	2.01 *	2.00 *	~1.96 *	1.96 *	2.04 m	1.96 m
H-16β	1.82 *	~2.05 *	1.74 *	1.79 *	~1.96 *	1.96 *	1.82 m	1.74 *
H-17	2.28 dd *9.9; 8.4*	2.42 dd *9.0; 8.5*	2.42 dd *9.5; 8.5*	2.42 d *9.6; 8.6*	2.49 dd *9.5; 8.5*	2.50 dd *9.5; 8.8*	2.46 dd *9.5; 8.7*	2.39 dd *9.5; 8.0*
H-22	3.76 dd *10.5; 1.7*	4.18 d *9.40*	3.45 dd *10.5; 1.7*	3.47 dd *9.4; 2.0*	2.85 d *2.4*	2.92 d *2.3*	3.58 dd *10.6; 1.8*	3.33 dd *11.0; 1.7*
H-23a	1.72 dd *14.7; 1.7*	1.80 dd *14.8; 9.4*	1.53 *	1.86 ddd *14.4; 4.2; 2.0*	2.72 dd *7.8; 2.4*	2.88 dd *7.6; 2.3*	2.39 bd *14.5*	1.28 m *13.0;11.5;11;4.6*
H-23b	1.44 dd *14.7;10.5*	1.56 bd *14.8*	1.09 ddd *14.1;10.0;4.6*	1.05 *	--	--	2.14 dd *14.5; 10.6*	1.66 m *13;12;4.2;1.8*
H-24a	--	--	1.67 *	1.65 dp *4.2; 7.0 (4x)*	1.12 m	1.24 dq *7.6; 7.0 (3x)*	--	1.78 *
H-25	1.89 h *6.8*	1.74 h *6.8*	1.78 dh *6.8 (6x); 3.4*	--	1.68 m	--	--	--
Me-18	0.879 s	0.871 s	0.874 s	0.874 s	0.843 s	0.844 s	0.885 s	0.892 s
Me-19	1.057 s	1.051 s	1.056 s	1.056 s	1.055 s	1.055 s	1.060 s	0.968 s
Me-21	1.226 s	1.280 s	1.197 s	1.211 s	1.306 s	1.319 s	1.256 s	1.204 s
Me-26	0.980 d *6.8*	0.968 d *6.8*	0.935 d *7.0*	1.142 s	0.987 d *6.8*	1.233 s	1.376 s	1.199 s
Me-27	0.912 d *6.8*	0.934 d *6.8*	0.801 d *6.8*	1.195 s	0.962 d *6.8*	1.230 s	1.323 s	1.191 s
Me-28	1.081 s	1.135 s	0.851 d *7.0*	1.034 d *7.0*	0.987 d *6.8*	1.019 d *7.0*	--	--

* The proton chemical shift was determined from 2D-COSY spectrum; [a] O-CH(O)-C_6H_4OH: 5.73 s, 6.77 m, 7.29 m;
[b] exomethylene protons: 5.14 bs, 4.96 bs; [c] H-11α: 1.81 m, H-24b: 1.44 ddd, J=13.0; 12.0, 4.0 Hz.

Table 2. Carbon-13 Chemical Shifts of Ecdysteroids **1 - 8** from *Paxillus atrotomentosus* in CD_3OD

Carbon	Chemical shifts [ppm]							
	1	**2** [a]	**3**	**4**	**5**	**6**	**7**	**8**
C-1	39.06	39.09	39.08	39.07	39.09	39.06	39.08	37.36
C-2	68.92	68.96	68.95	68.94	68.95	68.93	68.96	68.70
C-3	68.55	68.58	68.54	68.56	68.58	68.56	68.59	68.52
C-4	33.26	33.31	33.31	33.28	33.30	33.30	33.28	32.86
C-5	52.76	52.79	52.78	52.78	52.78	52.76	52.78	51.79
C-6	206.58	206.71	206.74	206.72	206.71	206.66	206.72	206.45
C-7	122.88	122.89	122.68	122.78	122.71	122.71	122.77	122.13
C-8	165.41	165.32	165.89	165.70	165.70	165.67	165.71	167.97
C-9	42.93	42.92	42.94	42.93	42.96	42.95	42.94	35.09
C-10	39.93	39.88	39.88	39.90	39.89	39.89	39.90	39.26
C-11	69.46	69.44	69.54	69.50	69.47	69.46	69.51	21.50
C-12	43.68	43.43	43.76	43.75	43.52	43.52	43.77	32.51
C-13	c	c	c	c	c	48.49	c	c
C-14	85.04	85.04	84.76	84.95	84.69	84.72	84.93	85.23
C-15	31.90	31.84	31.81	31.83	31.84	31.84	31.85	31.78
C-16	21.46	22.67	21.55	21.61	21.88	21.88	21.56	21.50
C-17	49.97	50.72	50.17	50.20	54.26	54.29	50.30	50.53
C-18	18.85	18.54	18.85	18.85	18.75	18.78	18.89	18.05
C-19	24.64	24.62	24.60	24.60	24.60	24.61	24.60	24.40
C-20	77.72	85.73	77.86	77.96	72.80	72.77	77.72	77.90
C-21	20.66	23.52	20.75	20.66	24.01	23.99	20.98	21.05
C-22	74.00	81.30	75.50	77.88	66.75	66.97	78.02	78.42
C-23	41.20	39.38	37.50	35.12	59.93	54.47	34.59	27.34
C-24	76.25	75.23	36.68	44.42	43.11	47.56	155.34	42.40
C-25	37.32	39.55	30.37	74.07	34.42	72.93	73.63	71.29
C-26	17.32	18.17	16.21	28.19	20.85	28.03	30.21	29.70
C-27	18.85	17.52	15.70	25.86	19.90	27.04	29.76	28.95
C-28	22.11	22.13	21.58	16.89	13.94	12.42	110.42	--

[a] O-CH(O)-C_6H_5-OH: 105.26, 131.22, 129.45 (2), 115.86 (2), 159.39; [c] overlapped with intensive multiplet of solvent (~ δ 49.00).

Table 3. Retention times [in min.] of ecdysteroids from *P. atrotomentosus* at various HPLC conditions

Compound	A	B	C
paxillosterone (**1**)	42.0	26.2	47.0
paxillosterone 20,22-p-BzOH acetal (**2**)	57.2	20.3	
atrotosterone A (**3**)	51.7	14.2	
25-hydroxyatrotosterone A (**4**)	41.1	50.5	
atrotosterone B (**5**)	56.3	17.7	
25-hydroxyatrotosterone B (**6**)	41.9	69.2	77.8
atrotosterone C (**7**)	41.1	42.6	
20-hydroxyecdysone (**8**)	42.7	31.7	47.5

A: RP; Separon SIX C18; gradient 10-70 % methanol in water / 50 min; 0.6 ml / min
B: NP; Silasorb 600; diethylether-acetonitril-water (78:19:3 v/v/v); 0.8 ml / min
C: NP; Silasorb 600; diethylether-hexane-methanol-water (44:43:12:1); 0.8 ml / min

Extraction and isolation of ecdysteroids.

Mushrooms *Paxillus atrotomentosus* (Batsch) Fr., were collected in forests at Příbram (central Bohemia). Fresh mushrooms (6650 g) mixed with dry ice (cca 10 kg) were in frosen form grounded and extracted with methanol (5 x 5000 ml). The combined extracts were concentrated to a reduced volume (1000 ml) and this solution was extracted with *n*-butanol (10 x 250 ml). The butanolic portion was evaporated to give 97.5 g of dry extract. The extract was fractionated by chromatography on neutral Al_2O_3 (400 g, deactivated with 10% of water) with a mobile phase containing ethylacetate-methanol mixtures (starting from 5% methanol in ethylacetate gradually increesing in 2 l solvent steps up to 95% methanol in ethylacetate. Collected 26 fractions (500 ml each) were monitored by a RP-HPLC. Fraction (16 - 19) containing ecdysteroids (840 mg) were further separated by a RP-HPLC using an 8x500 mm column packed with Separon SIX C-18, 5 μm (Laboratorní přístroje, Praha) and a methanol-water mobile phase at linear gradient from 18% to 80% of methanol during 200 min at flow rate 1.2 ml/min (system I). Over 90 fractions were collected. Compound **6** (4 mg) was obtained directly from the fraction 56 after evaporation of the solvent. Pairs of compounds **1, 8** (fr. 59) and **4, 7** (fr. 53) which co-eluted under these conditions and impurities containing compounds **2** and **5** (in fr. 83 and 82 respectively) were further separated and purified on NP-HPLC using column 8 x 500 mm packed with Silasorb 600, 5 μm (Lachema, Brno). As a mobile phase was used either diethylether-acetonirile-water (78:19:3 v/v/v), (system II), or for compound **3** (fr. 74) hexane-isopropanol-water (74:24:2 v/v/v), (system III), with a flow rate 2 ml/min in both cases. Pure compounds:**1** (356 mg), **2** (5 mg), **3** (41 mg), **4** (12 mg), **5** (4 mg), **6** (4 mg), **7** (5 mg) and **8** (41 mg) were obtained. Characteristic HPLC data recorded using analytical column are summarized in Table 3.

Paxillosterone (1)

Compound **1** (356.4 mg) was obtained in crystalline form (m.p. 219 - 225° C). Composition $C_{28}H_{46}O_8$, M.W.: 510 (by HR-MS). IR, ν_{max}: 3420 (O-H), 1650 (C=O) cm^{-1}. UV (in EtOH), λ_{max} (log ε): 243 (4.07). EI-MS, m/z (relat. intensity in %): 474 (8), 456 (39), 438 (41), 420 (31), 413 (54), 361 (28), 343 (57), 325 (28), 299 (21), 267 (32), 213 (21), 185 (18), 171 (23), 143 (17), 95 (24), 71 (44), 69 (41), 55 (36), 43 (100). FAB-MS, m/z (%): 533 [M+Na] (3), 511[M+H] (96), 493 [M+H-H_2O] (100), 475 [M+H-2H_2O] (55),457 [M+H-3H_2 O] (39), 441 (15), 439 (15), 391 (13), 373 (28), 345 (29), 327 (19), 317 (24), 299(29), 266 (19), 249 (27), 225 (24), 213 (23), 189 (23), 178 (28), 151 (64) 139 (41); HR-MS for $C_{28}H_{47}O_8$ [M+H] calculated 511.3271, found 511.3494; for $C_{28}H_{46}O_8Na$ [M+Na] calculated 533.3090 found 533.3201. For ^1H and ^{13}C NMR data see Tables 1 and 2.

Paxillosterone 20,22-acetonide (1a)

Toluene sulfonic acid (approx. 0.1 mg) was added to a solution of paxillosterone (**1**, 1.2 mg, 2.35 μmol) in acetone (200 μl). Reaction mixture was stirred for 40 min at room temperature. Pyridine (50 μl) was added and the mixture was evaporated. Residue dissolved in methanol (100 μl) was left to equilibrate. 20,22-Acetonide (1.2 mg, 93%) was obtained as sole product after NP-HPLC purification on Separon SGX, 7 mm, 8x250 mm, with CH_2Cl_2 - MeOH - H_2O (900:100:2 v/v/v), 4 ml/min, t_R 15.7 min. For ^1H and ^{13}C NMR data see Table 4.

Paxillosterone 20,22- and 22,24-phenylboronates (1b,1c)

Phenyl boronic acid (1.6 mg, 13.1 μmol, 1.6 equivalents) was added to a solution of paxillosterone (**1**, 4.1 mg, 8.0 μmol) in methanol (100 μl) and the mixture was stirred for 40 min at room temperature. The reaction mixture was evaporated and the dry residue was purified by NP-HPLC on Separon SGX, 7 mm, 8x250 mm, with CH_2Cl_2 - MeOH - H_2O (900:100:2 v/v/v), 4 ml/min, t_R 10.6 min., to give boronates (**1b,1c**, 3.65 mg, 76%). ^1H and ^{13}C NMR data see Table 4.

2,3,11-Triacetates of paxillosterone 20,22- and 22,24-phenylboronates (1d,1e)

Acetic anhydride (200 µl) was added to a solution of phenylboronates (**1b, 1c**, 3 mg, 5.0 µmol) in pyridine (200 µl). The reaction mixture was stirred for 2 hr at room temperature. Ethanol (3 ml) was then added and the whole mixture was evaporated. The mixture was purified by NP-HPLC on Separon SGX, 7 mm, 8x250 mm, with hexane-isopropanol-water (100:15:0.5 v/v/v), 4 ml/min, t_R 11.1 min., to give 2,3,11-triacetates (**1d, 1e**, 2.8 mg, 77%). ^{1}H NMR data see Table 4.

Table 4. Proton and Carbon-13 Chemical Shifts of Paxillosterone Derivatives **1a - 1e**

	Proton NMR					Carbon-13 NMR	
Proton	**1a** [a] (CD_3OD)	**1b + 1c** (~1:1) [b] (CD_3OD)	**1d + 1e** (~1:1) [c] (CD_3OD)	**1d + 1e** (~1:4) [d] ($CDCl_3$)	**Carbon**	**1a** [e] (CD_3OD)	**1b + 1c** (~1:1) [f] (CD_3OD)
H-1α	2.59 dd	2.61; 2.58	1.76	~1.71	**C-1**	39.09	39.11
H-1β	1.37 *	1.40; 1.38	1.64 dt	~1.62	**C-2**	68.90	68.94
H-2α	4.00 dt	4.01 dt	5.33	5.32	**C-3**	68.56	68.57
H-3α	3.95 bq	3.96 bq	5.39 bq	5.44 bq	**C-4**	33.21	33.26
H-4α	1.78 dt	1.78 *	1.98	~1.86	**C-5**	52.80	52.78
H-4β	1.68 *	1.69 *	1.76	~1.77	**C-6**	206.70	206.67
H-5	2.34 dd	2.35	2.34 dd	2.41 dd	**C-7**	122.80	122.89
H-7	5.80 d	5.83	5.93 m	5.94 d; 5.95 d	**C-8**	165.26	165.51
H-9	3.14 dd	3.18; 3.16	3.55 dd	3.44 dd	**C-9**	42.92	42.90
H-11β	4.09 ddd	~4.13	5.33 m	~5.32 m	**C-10**	39.86	39.94; 39.89
H-12α	2.20 dd	2.28; 2.27	~2.22	~2.11	**C-11**	69.44	69.46
H-12β	2.11 dd	2.18; 2.15	~2.37	2.40 dd	**C-12**	43.56	43.79
H-15α	1.95 *	~2.01	~2.05	~2.14	**C-13**	c	c
H-15β	1.58 *	~1.64	~1.70	~1.60	**C-14**	85.13	85.03
H-16α	2.04 *	~2.10	~2.09	2.24	**C-15**	31.80	31.90; 31.75
H-16β	1.93 *	~1.84	~1.88	1.79	**C-16**	21.36	21.68
H-17	2.28 t	2.52; 2.47	2.47 t	2.37 t; 2.31 t;	**C-17**	49.83	50.31
H-22	4.02 d	4.10; 4.58	4.08 dd; 4.57 bd	4.52 dd; 4.05 dd	**C-18**	18.54	19.13; 18.46
H-23a	1.67 dd	2.28; 2.08	2.26 dd; 1.81	1.80; 2.13 dd	**C-19**	24.62	24.60
H-23b	1.46 bd	1.51; 1.83	1.48 dd; 1.57	1.67; 1.41 dd	**C-20**	85.98	77.57; 84.96
H-25	1.70 *	2.01; 1.80	1.77; 1.99	1.92; 1.96	**C-21**	22.03	20.66
Me-18	0.817 s	0.916; 1.066	0.972; 1.058	1.042; 0.978	**C-22**	78.43	74.73
Me-19	1.056 s	1.066; 1.086	1.107; 1.124	1.142	**C-23**	43.56	41.17; 48.23
Me-21	1.172 s	1.281; 1.365	1.229; 1.320	1.331; 1.209	**C-24**	75.34	76.80; 88.17
					C-28	22.03	22.27

* The proton chemical shift was determined from 2D-COSY spectrum; [a] di-O-isopropylidene group: 1.397 s, 1.336 s; [b] >B-C_6H_5: 7.84 and 7.73 (*o*-), 7.35 and 7.31 (*m*-), 7.46 and 7.38 (*p*-); [c] >B-C_6H_5: 7.83 and 7.73 (*o*-), 7.35 and 7.31 (*m*-), 7.46 and 7.38 (*p*-); 3xOAc: 2.114 and 2.111; 1.970 and 1.981; 1.981 and 1.914 ; [d] >B-C_6H_5: 7.76 and 7.80 (*o*-), 7.38 and 7.36 (*m*-), 7.48 and 7.44 (*p*-); 3xOAc: 2.119 and 2.119; 1.998 and 1.994, 1.976 and 1.961; [e] $C(CH_3)_2$: 108.24, 29.26, 27.11; [f] >B-C_6H_5: 143.43 and 143.26 (α); 135.02 and 135.80 (*o*-), 128.42 and 128.79 (*m*-), 131.50 and 132.44 (*p*-).

Paxillosterone 20,22-p-hydroxybenzylidene acetal (2).

Compound **2** (5.5 mg) was obtained as amorphous solid. Composition $C_{35}H_{50}O_9$, M.W.: 614 (by HR-MS). IR, ν_{max}: 3409 (O-H), 1657 (C=O), 1617, 1518 (C=C), 1102 (C-O) cm^{-1}. EI-MS, m/z (intensity in %): 456 (1), 438 (3), 423 (2), 420 (1), 413 (3), 404 (2), 395 (3), 354 (7), 342 (10), 337 (6), 326 (14), 299 (21), 281 (10), 157

(26), 139 (19), 121 (96), 107 (30), 87 (70), 69 (68), 55 (68), 43 (100). FAB-MS, m/z (%): 637 [M+Na] (3), 615 [M+H] (2), 597 (2), 475 (7), 457 (5), 439 (3), 422 (8), 400 (20), 391 (4), 383 (2), 355 (4), 345 (2), 327 (3), 312 (6), 299 (4), 284 (4), 263 (7), 250 (7), 188 (17), 121 (39), 109 (61), 92 (36), 83 (42), 69 (72), 55 (100); HR-MS for $C_{35}H_{51}O_9$ [M+H] calculated 615.7439, found 615.7342; For ^1H and ^{13}C NMR data see Tables 1 and 2.

Atrotosterone A (3).

Compound **3** (41.0 mg) was obtained as amorphous solid. Composition $C_{28}H_{46}O_7$, M.W.: 494. IR, ν_{max}: 3412 (O-H), 1658 (C=O), 1050 (C - O) cm^{-1}. EI-MS, m/z (relat. intensity in %): 379 (4), 361 (18), 343 (100), 325 (66), 307 (12), 300 (6), 283 (6), 267 (43), 255 (4), 213 (5), 187 (7), 173 (6), 161 (3), 143 (3), 123 (5), 95 (5), 83 (5), 71 (7), 55 (4), 43 (14). FAB-MS, m/z (%): 517 [M+Na] (8), 495[M+H] (12), 477 (14), 459 (8), 441 (5), 423 (3), 399 (5), 345 (5), 329 (5), 317 (5), 299 (9), 281 (3), 249 (6), 225 (7), 149 (24), 129 (15), 113 (17), 95 (38), 83 (41), 71 (74), 55 (100); HR-MS for $C_{28}H_{47}O_7$ [M+H] calculated 495.3322, found 495.3334. For ^1H and ^{13}C NMR data see Tables 1 and 2.

25-Hydroxyatrotosterone A (4).

Compound **4** (11.9 mg) was obtained as amorphous solid. Composition $C_{28}H_{46}O_8$, M.W.: 510. IR, ν_{max}: 3404 (O-H), 1657 (C=O), 1054, 1072 (C - O) cm^{-1}. FAB-MS, m/z (relat. int.9 in %): 533 [M+Na] (4), 511 [M+H] (2), 493 (12), 475 (13), 457 (11), 439 (3), 387 (2), 379 (2), 373 (2), 362 (4), 345 (5), 317 (6), 299 (9), 281 (5), 249 (7), 225 (8), 213 (10), 197 (12), 181 (23), 157 (25), 113 (75), 93 (100), 83 (71), 69 (70), 57 (95); HR-MS for $C_{28}H_{46}O_8Na$ [M+Na] calculated 533.3090, found 533.3198. For ^1H and ^{13}C NMR see Tables 1 and 2.

Atrotosterone B (5).

Compound **5** (3.9 mg) was obtained as amorphous solid. Composition $C_{28}H_{44}O_7$, M.W.: 492. IR, ν_{max}: 3406 (O-H), 1657 (C=O), 1061 (C - O) cm^{-1}. EI-MS, m/z (relat. intensity in %): 456 [M-2xH_2O] (1), 438 (2), 409 (1), 395 (1), 367 (2), 343 (7), 325 (8), 309 (5), 300 (25), 285 (25), 267 (21), 185 (13), 171 (17), 157 (15), 139 (13), 129 (17), 118 (33), 83 (45), 71 (43), 55 (53), 43 (100). FAB-MS, m/z (%): 515 [M+Na] (8), 493 [M+H] (10), 475 (35), 457 (7), 439 (3), 316 (10), 299 (8), 283 (8), 281 (8), 250 (58), 231 (11), 213 (13), 133 (55), 115 (35), 105 (33), 91 (60), 83 (87), 71 (55), 55 (100); HR-MS for $C_{28}H_{45}O_7$ [M+H] calculated 493.3165, found 493.3261. For ^1H and ^{13}C NMR data see Tables 1 and 2.

25-Hydroxyatrotosterone B (6).

Compound **6** (4.2 mg) was obtained as amorphous solid. Composition $C_{28}H_{44}O_8$, M.W.: 508. IR, ν_{max}: 3386 (O-H), 1652 (C=O), 1589 (C=C) cm^{-1}. FAB-MS, m/z (%): 531 [M+Na] (4), 509 [M+H] (10), 491 (14), 473 (3), 455 (2), 397 (3), 373 (3), 345 (4), 291 (22), 249 (14), 213 (11), 189 (11), 161 (19), 145 (19), 109 (30), 91 (44), 81 (50), 69 (44), 59 (100); HR-MS for $C_{28}H_{45}O_8$ [M+H] calculated 509.3114, found 509.3104. For ^1H and ^{13}C NMR data see Tables 1 and 2.

Atrotosterone C (7).

Compound **7** (5.5 mg) was obtained as amorphous solid. Composition $C_{28}H_{44}O_8$, M.W.: 508. IR, ν_{max}: 3406 (O-H), 1657 (C=O), 1051, 1073 (C - O) cm^{-1}. FAB-MS, m/z (%): 531 [M+Na] (1), 509 [M+H] (3), 491 (2), 473 (3), 455 (4), 437 (2), 345 (5), 317 (7), 299 (12), 239 (18), 121 (49), 105 (51), 91 (87), 81 (63), 69 (70), 55 (100) HR-MS for $C_{28}H_{45}O_8$ [M+H] calculated 509.3114, found 509.3035. For ^1H and ^{13}C NMR data see Tables 1 and 2.

20-hydroxyecdysone (8)

From the RP-HPLC fraction 59 were separeted by NP-HPLC in system II paxillosterone (**1**) and compound **8** (41.1 mg) identified by analytical NP-HPLC in systemes A, B and C (Table 3) as 20-hydroxyecdysone, using authentic sample as internal standard. Composition $C_{27}H_{44}O_7$. HR-MS for $C_{27}H_{45}O_7$ [M+H] calculated 481.3165, found 481.3233. Identity was proven also by ^1H and ^{13}C NMR spectroscopy (see data in Tables 1 and 2).

Acknowledgement: We wish to thank Dr. F. Kotlaba for his help with the collection and identification of mushrooms, Dr. J. Kohoutová for MS and Dr. S. Vašíčková for IR spectra. Financial support by the Grant Agency of the Academy of Sciences of the Czech Republic for the project No. 45513 is acknowledged.

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